Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the *daf-2* insulin-like signaling pathway

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***C. elegans* insulin-like signaling regulates metabolism, development, and life span. This signaling pathway negatively regulates the activity of the forkhead transcription factor DAF-16. *daf-16* encodes multiple isoforms that are expressed in distinct tissue types and are probable orthologs of human FKHRL1, FKHR, and AFX. We show that human FKHRL1 can partially replace DAF-16, proving the orthology. In mammalian cells, insulin and insulin-like growth factor signaling activate AKT/PKB kinase to negatively regulate the nuclear localization of DAF-16 homologs (reviewed in [1]). We show that the absence of AKT consensus sites on DAF-16 is sufficient to cause dauer arrest in *daf-2(+)* animals, proving that *daf-16* is the major output of insulin signaling in *C. elegans*. FKHR, FKRHL1, and AFX may similarly be the major outputs of mammalian insulin signaling. *daf-2* insulin signaling, via AKT kinases, negatively regulates DAF-16 by controlling its nuclear localization. Surprisingly, we find that *daf-7* TGF-β signaling also regulates DAF-16 nuclear localization specifically at the time when the animal makes the commitment between diapause and reproductive development. *daf-16* function is supported by the combined action of two distinct promoter/enhancer elements, whereas the coding sequences of two major DAF-16 isoforms are interchangeable. Together, these observations suggest that the combined effects of transcriptional and posttranslational regulation of *daf-16* transduce insulin-like signals in *C. elegans* and perhaps more generally.**

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Results and discussion

Insulin signaling in *C. elegans* regulates the developmental decision to grow reproductively or arrest at the dauer stage, and it affects metabolism, stress resistance, fertility, and life span. Because strong loss-of-function mutations in *daf-16* can suppress all of the phenotypes caused by mutations in *daf-2* insulin/IGF receptor tyrosine kinase [2], temporally and spatially regulated expression of *daf-16* may be important to diversify the biological outputs of insulin signaling. The *daf-16* locus encodes three transcripts: a1, a2, and b, generated by alternative splicing and probable alternative promoters [3]. The predicted protein products of *daf-16a1* (DAF-16A1) and *daf-16a2* (DAF-16A2) are closely related, differing by only two amino acids, which is due to alternative splicing in exon 3. In contrast, *daf-16a* (refer to *a1* and *a2* together) and *daf-16b* share the C-terminal 319 amino acids encoded by common exons but differ in their amino-terminal coding regions; upstream from the common domain, DAF-16A1 (191 residues encoded by exon 1–4) and DAF-16B (211 residues encoded by exon 5) are only 32% identical. The 5′ ends of the *daf-16a* and *daf-16b* transcripts are separated by 8.4 kb, strongly supporting distinct promoters and possibly distinct enhancers for each transcript. A 6.4-kb segment from 5′ to *daf-16a* directs expression in body wall muscles, hypodermis, and intestine [3], whereas a 6.1-kb segment from 5′ to *daf-16b* directs expression in the pharynx (S. Ogg and G.R., unpublished data). Thus, there may be distinct *cis*-regulatory regions that control the expression of each isoform. Because of their distinct expression patterns and N-terminal coding sequences, *daf-16a* and *daf-16b* could mediate distinct functions.

We tested this possibility by analyzing the phenotype of *daf-16(m26)* and *daf-16(mg54)*, predicted to specifically affect *daf-16a* [3, 4]. *daf-16(m26)* carries a mutation in the splice donor site of intron 2, whereas *daf-16(mg54)* carries a nonsense mutation in exon 3, both of which are specific to *daf-16a*. We tested the ability of these *daf-16a*-specific mutants to form dauers constitutively (dauer arrest constitutive) or to live long as adults (ageing alteration) in a *daf-2(e1370)* mutant background. When *daf-16* is wild-type, *daf-2(e1370)* animals are nearly 100% dauer arrest constitutive at 25°C [5]. When animals of this genotype are grown at 15°C until the L4 stage and are then shifted to 25°C, the adults have an average life span of 30 days, about twice that of wild-type ([6] and Figure 1). The *daf-16(mgDf47)* null mutation, which deletes exon 5 through the end of the coding region and therefore lacks the DNA binding domains of both DAF-16A and DAF-16B [3], fully suppresses both the dauer arrest and longevity phe-

Table 1

Dauer arrest phenotype of *daf-16(x); daf-2(e1370)* mutants.

X =	Reproductive development	Dauer(-like) arrest	Other
mgDf47	96.5% (734)	0.1% (1)	3.4% (26)
mg54	92.3% (515)	0.2% (1)	7.5% (42)
m26	96.0% (1086)	2.1% (24)	1.9% (21)
+	0%	75.5% (163)	24.5% (53)

Synchronized (within 3 hr) progeny of gravid mutant mothers were shifted to the test temperature (25°C) for 46–52 hr. They were then scored for the dauer arrest constitutive phenotype visually. For all tables, reproductively developing animals included L4 and adult animals. Dauer(-like) arrested animals were either complete or partial dauers (showing one or more features, such as radial constriction, dauer alea, and pharyngeal constriction) or L2d animals. "Other" includes animals that were of L1, L2, or L3 stages that had no dauer features. The number of animals is in parentheses. Distributions of arrested animals between the "dauer(-like)" and the "other" classes were somewhat variable from experiment to experiment. However, the combined percentage of the two classes was quantitatively reproducible for a given genotype. Strains used were: GR1309 for *daf-16(mgDf47); daf-2(e1370)*, GR1308 for *daf-16(mg54); daf-2(e1370)*, GR1117 for *daf-16(m26); daf-2(e1370)*, and GR1122 for *daf-2(e1370)*.

notypes of a *daf-2* mutant: *daf-16(mgDf47); daf-2(e1370)* double mutant animals form virtually no dauers under replete conditions at 25°C, and the adults live even shorter than wild-type ([5]; Table 1 and Figure 1). Under similar conditions, few *daf-16(mg54); daf-2(e1370)* animals (1/558 assayed) or *daf-16(m26); daf-2(e1370)* animals (24/1131 assayed) arrest at the dauer stage (Table 1). Similarly, neither *daf-16(m26); daf-2(e1370)* nor *daf-16(mg54); daf-2 (e1370)* animals lived longer than wild-type control animals (Figure 1). Thus, *daf-16a*-specific mutations behave similarly to *daf-16(null)* in their ability to suppress *daf-2 (e1370)*. These results indicate that the *daf-16b* activity that remains in these *daf-16a* probable null mutants is not sufficient to supply *daf-16* gene function.

The ability of a *daf-16a* mutation to fully suppress both the aging and dauer arrest phenotypes of *daf-2* strongly suggests that *daf-16a* is a major target of *C. elegans* insulin signaling. Alternatively, animals depend on the function of both *daf-16a* and *daf-16b*; the loss of either one may be sufficient to abrogate *daf-16* function. There is no *daf-16b*-specific lesion in our large collection of *daf-16* mutant alleles (in addition to previously published data in [3] and [4], we sequenced exon 5 in 12 mutants; data not shown), weakly supporting a model that *daf-16b* has a less-central function in dauer arrest than *daf-16a*.

We addressed the function of *daf-16b* using RNA interference (RNAi; [7]). The *daf-16a* and *daf-16b* exons that encode the distinct N-terminal regions are divergent enough that double-stranded RNA corresponding to *daf-16b* will not also target *daf-16a* (the longest uninterrupted run of nucleic acid identity is 5 base pairs between *daf-16b*-specific exon 5 used in RNAi and the entire

Figure 1

Adult life span of *daf-16; daf-2* double mutants. The life span of adult animals grown at 25°C presented as a Kaplan-Meier plot. *mgDf47* is a null allele of *daf-16*, whereas *mg54* and *m26* are alleles that specifically affect the *daf-16a* isoform [3]. Strains used were: Bristol N2 for wild-type (n = 592), GR1309 for *daf-16(mgDf47); daf-2(e1370)* (n = 99), GR1308 for *daf-16(mg54); daf-2(e1370)* (n = 104), GR1117 for *daf-16(m26); daf-2(e1370)* (n = 68), GR1122 for *daf-2(e1370)* (n = 40), and GR1329 for *daf-16(mgDf47)* (n = 229). The asterisk indicates that the *daf-2(e1370)* life span was determined in slightly different conditions than the rest, on regular rather than FUdR-containing (fluorodeoxyuridine) plates. FUdR does not affect the life span of animals [20].

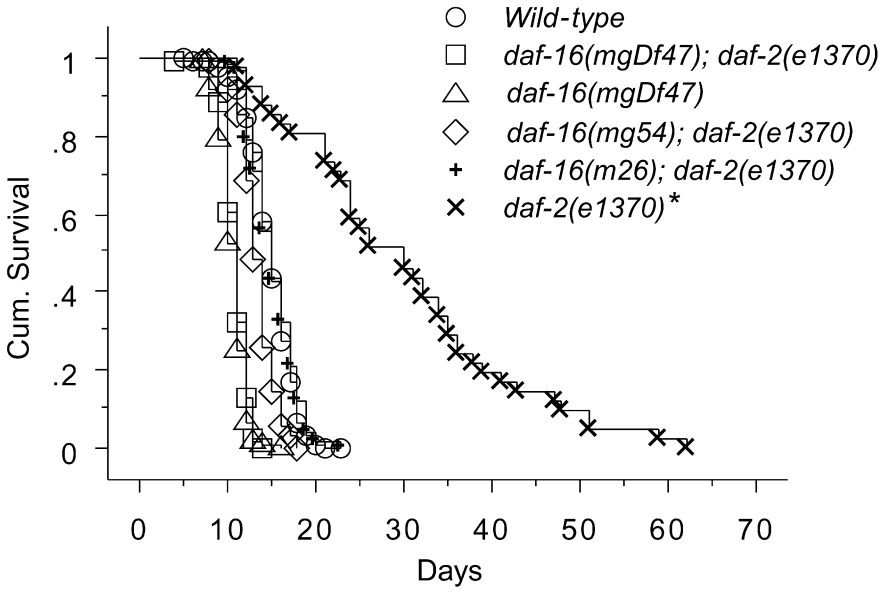
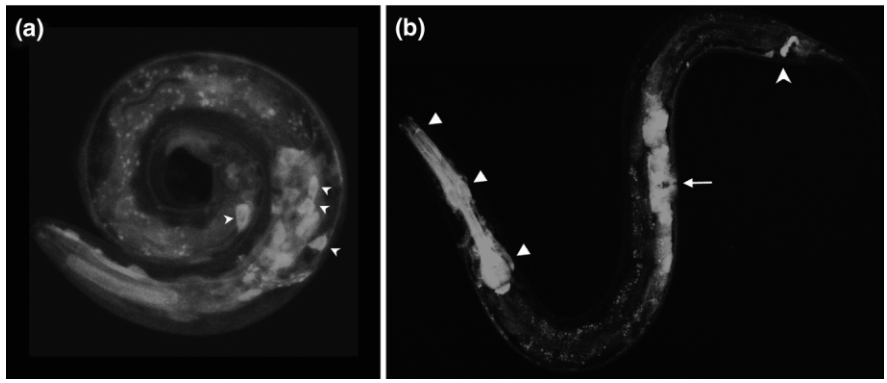


Figure 2



GFP expression driven by a *daf-16*β enhancer/promoter element. Fluorescent micrographs of *daf-16(mgDf47)* animals that carry a *daf-16*-rescuing translational GFP fusion *Ex*[*daf-16*β::GFP::DAF-16B] transgene. **(a)** An L1 animal showing GFP expression in neurons; four fluorescent neurons are marked by arrowheads. **(b)** A late L4 animal. GFP was expressed in the pharynx (marked by triangles), somatic gonad (with an arrow pointing to the vulva), and in neurons in the tail (marked by an arrowhead). Transgene array *Ex*[*daf-16*β::GFP::DAF-16B] was made as a complex extrachromosomal array in order to get better stability of transgene expression [21]. GR1329 *daf-16(mgDf47)* animals were transformed with a mixture of PvuII-digested worm genomic DNA (100 μg/ml) and *daf-16*β::GFP::DAF-16B minigene (0.25 μg/ml, in the form of PCR products). Higher concentrations of *daf-16*β::GFP::DAF-16B drastically reduced the viability of transgenic embryos (data not shown). DNA templates used: R13H8, a genomic cosmid clone from A. Coulson, and pPD117.01, a GFP vector. PCR primer sequences and procedures for fusions are available at http://whitney.caltech.edu/~raymond/daf16.html or upon request.

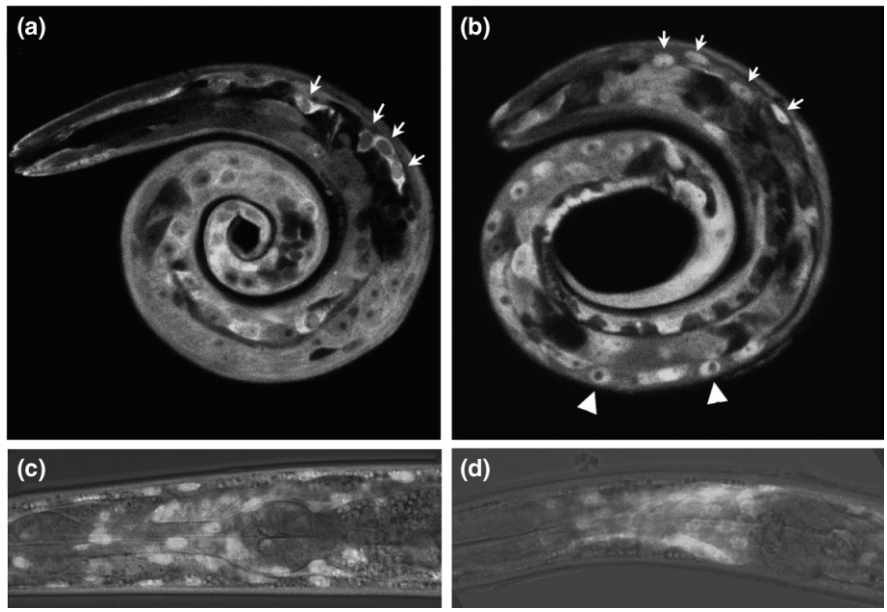
daf-16a1 transcript). RNAi experiments showed that inactivation of *daf-16b* in *daf-2(e1370)* had much weaker effects on dauer arrest than did the RNAi inactivation of *daf-16a* alone: progeny of *daf-2(e1370)* animals that received *daf-16b* dsRNA were 100% dauer arrest constitutive (44 of 44 animals), whereas progeny from mothers that received *daf-16a* dsRNA (corresponding to exons 1–4) were 0% dauer arrest constitutive (0 of 69 animals). The combination of *daf-16a*- and *daf-16b*-specific dsRNA was also effective in suppressing *daf-2(e1370)* dauer arrest (1 of 75 progeny became a dauer; 74 developed reproductively). These results showed that *daf-16a* is the major genetic activity from the *daf-16* locus for *daf-2*-mediated dauer arrest and longevity control.

What makes *daf-16a* functionally different from *daf-16b*? We first considered the possibility that the two isoforms are differentially expressed. By transgenic rescue experiments, we found that a 2-kb (12,727–14,813 bp in cosmid R13H8, accession number AF039717) genomic region immediately 5′ to the predicted first ATG codon of *daf-16b* was sufficient to direct expression of *daf-16b*-coding sequences to rescue the dauer arrest-defective phenotype of *daf-16*(*mgDf47*) ([3] and see below). We named this transcriptional regulatory element *daf-16*β. We analyzed the cellular expression pattern of a *daf-16*β-promoter::GFP::DAF-16B fusion gene, in which GFP was fused at the less well-conserved N terminus of DAF-16B. This fusion gene rescues the dauer arrest defective phenotype of *daf-16(mgDf47)* (see below). We first detected the functional GFP::DAF-16B fusion protein fluorescence in early embryos, prior to morphogenesis. After hatching and in all later developmental stages, transgenic larvae showed GFP in the pharynx and in many neurons throughout the body. From the late L3 stage onward, GFP was also detected in somatic gonads (Figure 2).

The *daf-16*α promoter element was defined by the 6.4-kb (48–6408 bp in cosmid R13H8) genomic region 5′ to the initiation ATG of *daf-16a*. We analyzed the expression pattern of a *daf-16*α-promoter::GFP::DAF-16B fusion gene, which similarly rescues the dauer arrest-defective phenotype of *daf-16(mgDf47)* (data not shown). GFP was first detected in comma-stage embryos. After hatching, transgenic animals showed high levels of GFP expression in almost all somatic cells. In contrast to the *daf-16*β fusion gene, little GFP was detected in somatic gonad or pharynx (except occasionally in one or two unidentified cells in the terminal bulb; Figure 3 and data not shown).

In order to validate the significance of these expression patterns, we tested these fusion genes bearing full DAF-16B protein-coding segments for the rescue of different aspects of the *daf-16* mutant phenotype. There are multiple features of dauer larvae that differ from reproductively developing L3 larvae: a constricted pharynx, a shift to fat storage, cuticular structures called dauer alae, as well as resistance to stress and an increased life span. We found that each of *daf-16*α and *daf-16*β promoter/enhancer DNA segments can direct expression of DAF-16 proteins to rescue some aspects of *daf-16* loss-of-function mutant phenotype, but their activities were partially complementary to each other (summarized in Table 2). *daf-16*(*mgDf47*) animals are partially dauer defective [8], unable to form complete, SDS-resistant dauers under inducing conditions (such as on starved plates), and they have a shorter life span than wild-type ([5]; Figure 1 and data not shown). We found that *daf-16*(*mgDf47*) transgenic animals bearing either *daf-16*α::DAF-16A1 or *daf-16*β::DAF-16B were able to form SDS-resistant dauer larvae on starved plates. However, animals carrying *daf-16*α::DAF-16A1 formed dauers with little pharynx remodeling, which is normally found in dauer animals (0

Figure 3



DAF-16 protein nuclear localization is dynamically regulated by *daf-2* and *daf-7*. Fluorescent micrographs of animals carrying *Ex*[*daf-16*α::GFP::DAF-16B], a *daf-16*/GFP fusion gene that can rescue *daf-16* mutant dauer-defective phenotype (data not shown). **(a)** An L1 animal of genotype *daf-16(mgDf47); daf-2(+).* GFP::DAF-16 is mostly cytoplasmic, with a perinuclear concentration. The soma of four neurons in the head are marked by arrows. **(b)** An L1 animal of genotype *daf-16(mgDf47); daf-2(e1370).* GFP::DAF-16 is prominently nuclear localized. The nuclei of four neurons in the head and two hypodermal cells in the midbody are marked by arrows and triangles, respectively. **(c)** The head region of a *daf-16(mgDf47); daf-7(m62)* L2d predauer-stage animal. GFP::DAF-16 was almost exclusively localized in nuclei throughout the animal. **(d)** The head region of a *daf-16(mgDf47); daf-7(m62)* dauer animal carrying the transgene showing a more diffuse, primarily perinuclear GFP::DAF-16 localization. *daf-16*α-promoter::GFP::DAF-16B fusion PCR product (concentration at 2.5 ng/μl) was mixed with 98 ng/μl pRF4 [14] *rol-6* DNA and was used to transform GR1329 *daf-16*(*mgDf47*) animals. The stably transmitting transgene was then crossed into *daf-16(mgDf47); daf-2(e1370)* and *daf-16(mgDf47); daf-7(m62)* mutants.

of 30 SDS-resistant dauers examined had a constricted pharynx), whereas dauers that carried *daf-16*β::DAF-16B had more complete pharyngeal constriction (11 of 17 dauers examined). This result is consistent with an autonomous DAF-16 protein function in the pharynx, because the *daf-16*β promoter element is active in the pharynx, whereas *daf-16*α is not.

We also found a difference between the life span regulatory activity of the two fusion genes. *daf-16(mgDf47); Ex*[*daf-16*α::DAF-16A1] transgenic animals had an average adult life span 65% longer that of control *daf-16(mgDf47)* animals, whereas *Ex*[*daf-16*β::DAF-16B] transgenic animals lived, on the average, only 14% longer than the control (Table 2).

Table 2

Comparisons of *daf-16*α and -β promoter-driven fusion gene activity.

Transgene	Phenotype in *daf-16(mgDf47)*		
	SDS-r	Pha const.	Mean lifespan (N)
Marker only	0/3 lines	n/a	78% (40), 96% (39)[a]
*daf-16*α::DAF-16A1	2/3 lines	0/30 dauers	165% (40)
*daf-16*α::DAF-16B	2/3 lines	0/29 dauers	155% (40)
*daf-16*β::DAF-16A1	3/3 lines	15/25 dauers	114% (40)
*daf-16*β::DAF-16B	2/2 lines	11/17 dauers	114% (40)

SDS-r: presence of 1% SDS-resistant dauers on starved plates. Each line is an independently generated transgenic strain. Pha const.: pharyngeal constriction in SDS-resistant dauers. Lifespan: mean adult lifespan measured at 25°C, compared with *daf-16(mgDf47)* without transgene.

[a]Two independent lines showing different population lifespans.

*daf-16*α-promoter::DAF-16A1, *daf-16*α-promoter::DAF-16B, *daf-16*β-promoter::DAF-16A1, and *daf-16*β-promoter::DAF-16B fusion PCR products (concentration at 2.5 ng/μl) were each mixed with 50 ng/μl pRF4 and 50 ng/μl pTG96 (expresses GFP in every nucleus, except, germ line [22]) plasmid DNA and were used to transform GR1329 *daf-16(mgDf47)* animals. Transgenic lines were also made with markers only to serve as controls. DNA templates used in making PCR fusions: R13H8, a genomic cosmid clone from A. Coulson; pdaf16a1, a *daf-16a1* cDNA clone described previously [3]; pdaf16b, a cDNA clone isolated in this study that contains the ORF part of AF020344 [3].

Given the fact that DAF-16A and DAF-16B proteins have very different sequences at their N termini, we tested if the differences in coding sequences contribute to the difference in function of the fusion genes. By swapping the protein-coding segments between the genes to generate *daf-16*α::DAF-16B and *daf-16*β::DAF-16A1 fusion transgenes, we found that the protein-coding sequences were essentially interchangeable for life span and dauer arrest regulation; no significant differences were detected when comparing fusion genes with the same promoter element but different coding sequences (Table 2). Thus, at least in the context of these fusion genes, transcriptional rather than protein sequence differences subserve the particular biological functions of *daf-16*.

Our genetic and fusion gene analyses indicate that *daf-16a* encodes the most important protein products of the *daf-16* locus and suggest that the distinction between the *daf-16a* and *daf-16b* gene activities is likely based on their distinct expression domains rather than the differences between the DAF-16A and DAF-16B proteins (Figure 1 and Table 2). Although we did not compare the expression levels of the *daf-16* promoter fusion genes, the fusion genes were injected at similar low-copy injection concentrations (2.5 ng/μl), and multiple transgenic lines were tested. We therefore favor the model that the *daf-16*α promoter activity controls aging, whereas the *daf-16*β promoter activity is necessary for pharyngeal restructuring during dauer arrest. Both promoters contribute to dauer arrest. It may be that *daf-16*α and *daf-16*β promoters supply combined trascriptional regulation more to *daf-16a* than to *daf-16b* to cause dauer arrest and long life span. However, our transgenic analyses may have exaggerated the role of *daf-16*β promoter in dauer formation; chromosomal expression of *daf-16b* alone may not be sufficiently high to significantly effect dauer formation. Furthermore, we cannot rule out that expression of *daf-16b* is also affected in *daf-16(mg54)* and *daf-16(m26)* mutants. Further quantitative analyses of expressed RNA species in different genetic backgrounds will resolve these issues.

Mammalian AFX, FKHR, and FKHRL1 are closely related in sequence to worm DAF-16. These mammalian genes are expressed in distinct tissue types and have been implicated in insulin and insulin-like growth factor signaling pathways (reviewed in [1]). To prove the orthology between *daf-16* and these related genes, we tested the ability of human FKHRL1 to substitute for DAF-16 in the functional fusion genes described above. We found that a *daf-16*β::FKHRL1 fusion gene supplies *daf-16* gene activity to a *daf-16(mgDf47); daf-2(e1370)* double mutant (Table 3). Under restrictive conditions, animals that carried a *daf-16*β::FKHRL1 fusion gene showed significantly higher levels (>70%) of *daf-2* mutant-like dauer and early larval arrest compared to the nontransgenic controls (3%). Under similar conditions (except at lower injection concentrations), the *daf-16*β::DAF-16B fusion gene showed 100% arrest. However, most arrested animals bearing *daf-16*β::FKHRL1 showed no or partial dauer features, such as body radial constrictions, dauer alae, and pharyngeal constrictions. We conclude that human FKHRL1 can function like DAF-16 in mediating insulin signaling in *C. elegans*, but that its activity is weak.

Mammalian FKHRL1 is negatively regulated by AKT/PKB kinases [9]. *C. elegans akt-1* and *akt-2* have been shown genetically to negatively regulate *daf-16* [10]. On DAF-16 (either the A or B form), there are four sites that conform to the consensus of mammalian AKT phosphorylation. To address the biological importance of AKT phosphorylation, we assayed the phenotypic effects of expressing the AKT phosphorylation-defective DAF-16A1-4A mutant [11] in the *daf-16*(*mgDf47*) background. In the anticipation that transgenic animals might arrest constitutively as dauers, we isolated the transgenic animals after microinjection by feeding the injected parents and offspring on *Eschericia coli* that express *daf-16* dsRNA to keep the DAF-16 expression levels low as lines were established [12]. Phenotype assays were then done on the decendents of those animals now feeding on non-*daf-16* RNAi *E. coli* for one generation or two.

We found that *daf-16*(*mgDf47*) animals bearing the *daf-16*α::DAF16A1-4A fusion gene showed moderate (~60%) to nearly complete (99%) constitutive dauer or otherwise larval arrest under nondauer-inducing conditions, depending on whether they were the first or second generation progeny of animals that were fed *daf-16* dsRNA-expressing bacteria (Table 4). This result indicates that DAF-16 phosphorylation on some or all four S/T residues by AKT (or other related) kinases is a crucial aspect of *daf-16* gene function and a pivotal point of regulation. We could not measure the adult life span of these transgenic animals because the variably penetrant arrest phenotype interfered with the isolation of a synchronized adult population. Because decrease in *akt-1* and *akt-2* gene activity causes a similar phenotype to the inactivation of AKT phosphorylation sites on DAF-16 [10], we favor the model that AKT-1 and AKT-2 are the major inputs of DAF-16 at these sites.

In contrast to our findings, Lin et al. reported that a *daf-16A-4A* mutant expressed from a transgene rescued a *daf-16*(null) mutant but did not cause any constitutive dauer arrest or longevity phenotype in a *daf-2(+)* genetic background. They concluded that *daf-2* regulates *daf-16* via a non-AKT consensus site-based mechanism [13]. We consider three possibilities to reconcile the difference between their and our observations. First, the expression levels of the transgenes may be significantly different. Since they did not use RNAi to inhibit any dauer arrest phenotypes in the initial line generation by transforma-

Table 3

Rescue of the *daf-16(mgDf47); daf-2(e1370)* dauer-defective phenotype by human FKHRL1.

Transgene	Reproductive development (N)	Dauer(-like) arrest (N)	L3/L2 (N)	L1/egg (N)
No transgene control	97.2% (141)	0.7% (1)	1.4% (2)	0.7% (1)
*daf-16*β::DAF-16B	0%	48% (12)	20% (5)	32% (8)
*daf-16*β::FKHRL1(line #1)	29.0% (31)	31.8% (34)	1.9% (2)	37.4% (40)
*daf-16*β::FKHRL1(line #2)	9.5% (8)	47.6% (40)	17.9% (15)	25.0% (21)

Animals were scored 51 hr after egglay, at 25°C. The PCR product of *daf-16*β*-promoter*::FKHRL1 (10 ng/μl) was mixed with pRF4 (45 ng/μl) and pTG96 (45 ng/μl) and was used to transform GR1309 *daf-16(mgDf47); daf-2(e1370) animals*. A template used in the PCR involving human FKHRL1 was pHs-FKHRL1-HA, a HA-tagged human FKHRL1 cDNA plasmid, a gift from M.J. Anderson and K.C. Arden (AF032886, [23]).

tion, it is possible that their transgenes were selected to express at lower levels. Indeed they reported transgene toxicity (see Methods in [13]). Second, their construct contains extensive *daf-16* intronic sequences that are not present in our construct. For example, intron 5 contains the β promoter element (this report). These sequences may affect the temporal and spatial patterns of gene expression. Third, Lin et al. expressed a GFP fusion protein. It may be that the GFP::DAF-16 fusion is functionally different from the native protein. We do not favor the model that overexpression of *daf-16*α::DAF16A1-4A causes constitutive dauer arrest. Control transgenes that were produced by the same injection concentration (2.5 ng/μl) but that expressed the wild-type DAF-16A1 proteins did not induce dauer arrest (Table 4). We have attempted to produce high levels of *daf-16* expression using a heat shock promoter construct. Transgenic animals carrying an integrated transgene of *hsp16-2*::DAF-16B arrested as partial or complete dauers only when subjected to repeat, periodic heat shock throughout their lives (unpublished data). This result argues that it is not easy to force expression of wild-type DAF-16 to a level sufficient to cause a dauer arrest constitutive phenotype.

The expression of mutant DAF-16 from the transgene suffers from some limitations. First, expression of *Ex[daf-16*α*::DAF16A1-4A]* is heavily selected against because of the arrest and lethality it causes. We could only maintain the stock by feeding them with *daf-16* RNAi bacteria. This RNAi treatment was not effective enough to completely shut down transgene expression (Table 4 and data not shown). Furthermore, there appears to be RNAi perdurance; the RNA interference of *daf-16* took more than one generation to wear off completely after transfer of animals from feeding RNAi bacteria (Table 4 and data not shown). Third, extrachromosomal transgenes tend to be lost during mitotic divisions, causing mosaicism and reducing overall expression [14]. This may explain why the *daf-16*α::DAF16A1-4A transgenic animals failed to precisely phenotype copy *daf-2(null)* mutant animals.

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Table 4

Dauer arrest phenotype caused by AKT phosphorylation incompetent DAF-16.

Transgene[a]	Reproductive development (N)	Dauer(-like) arrest (N)	L3/L2 (N)	L1/egg (N)
DAF-16(4A)[b]	1.1% (1)	25.8% (23)	58% (52)	14.6% (13)
No Transgene siblings[b]	94.9% (74)	0%	3.8% (3)	1.3% (1)
DAF-16(4A)[c]	44.2% (76)	19.2% (33)	36.6% (63)	n/c
DAF-16(WT)	94.0% (480)	0%	6% (33)	n/c

Animals were reared at 25°C and were scored 48.5–56 hr after egglay.
[a] All animals carried *daf-16(mgDf47)*. n/c means we had not particularly counted that class.
[b] Grandparents had been fed with *daf-16* dsRNA-expressing bacteria.
[c] Parents had been fed with *daf-16* dsRNA-expressing bacteria.
The DAF-16(WT) transgene was made by germline transformation with the PCR-generaged chimeric construct *daf-16*α::DAF-16A1 (2.5 μg/ml); DAF-16(4A) was made with *daf-16*α::DAF-16A1-4A (2.5 μg/ml) mutant fusion construct. The 4A mutant contains for S/T to A mutations that would abolish all potential sites of AKT phosphorylation [11]. *daf-16*α::DAF16A1-4A fusion transgenic animals were made by transforming GR1329 *daf-16(mgDf47)* animals with a mixture of 2.5 ng/μl *daf-16*α::DAF16A1-4A as a PCR product, 46 ng/μl pRF4, 34 ng/μl pTG96, and 11 ng/μl pPHgfp1 (expresses GFP in hypodermis [24]). Transgenic animals had to be maintained with *daf-16* RNAi *E. coli*. When grown on OP50 *E. coli*, transgenic animals are selected against by their dauer arrest phenotype. For feeding RNAi, a 970-bp PvuII/XhoI fragment common to all daf-16 isoforms was cloned into the SmaI site of plasmid pPD129.36, between T7 polymerase sites. The resulting plasmid, pT7-daf16common-T7, was used to transform *E. coli* strain HT115(DE3) and to make a bacterial strain, RX99. Feeding RNAi was performed on worm culture NG agar plates containing IPTG, ampicillin, and tetracyclin, as described by Timmons et al. [12].

DAF-16 homologous proteins by inhibiting nuclear import or activating nuclear export via AKT phosphorylation (reviewed in [1]). To see if this regulation is conserved in *C. elegans*, we assayed the subcellular localization of a functional GFP::DAF-16B fusion gene (driven by *daf-16*α promoter) in different *daf-2* genetic backgrounds. We found that, in *daf-16*(*mgDf47*)*; daf-2(+)* animals, GFP::DAF-16B was predominantly cytoplasmic, with a high concentration around the nucleus (Figure 3A). In contrast, in a *daf-16(mgDf47); daf-2(e1370)* mutant background, where upstream insulin-like signaling is defective at 25°C, GFP::DAF-16B was concentrated in the nucleus (Figure 3B). This change in subcellular localization of GFP::DAF-16 appeared from the embryonic stage through late adulthood. Therefore, our results indicate that DAF-16 and its mammalian homologs are regulated by similar mechanisms. These results are in agreement with those reported by Lin et al. [13].

Our results indicate that phosphorylation of DAF-16 on all or some of the four S/T residues within AKT phosphorylation consensus sites is the major means by which insulin-like signaling transduces to *daf-16*. Furthermore, our results indicate that the absence of phosphorylation of these AKT consensus sites on DAF-16 is sufficient, independent of insulin signaling, to induce dauer arrest. This proves that *daf-16* is the major output of insulin signaling in *C. elegans* and suggests that FKHR, FKRHL1, and AFX may similarly be the major outputs of mammalian insulin signaling. Our results also argue that the AKT signaling output of DAF-2, and perhaps the orthologous mammalian insulin-like receptor tyrosine kinases, is the key output, rather than the wide range of other signaling pathways that have been suggested by biochemical analyses.

The *daf-7* TGF-β signaling pathway also regulates dauer arrest (reviewed in [15]). Although mutations in the TGF-β pathway can enhance the dauer arrest constitutive phenotype caused by reductions in *daf-2* insulin-like signaling, there is no evidence that demonstrates a mechanistic coupling between these two signaling pathways [3]. Furthermore, although both pathways regulate dauer arrest, only the insulin-like, but not the TGF-β, pathway affects life span (reviewed in [16]).

We checked if decreased TGF-β signaling also regulates DAF-16 nuclear localization. It did, although in a different manner from the insulin pathway. In a *daf-16(mgDf47); daf-7(m62)* background under dauer-inducing conditions, GFP::DAF-16B was almost exclusively localized in the nucleus throughout the animal but only during the L2d predauer stage Figure 3c. The L2d stage is an extension of the normal second larval stage that only forms under dauer-inducing environmental conditions. L2d animals, compared to normal L2, are slightly larger, due to prolonged feeding, and darker, apparently due to storage granules in the intestine. In the middle of the L2d stage, the animal becomes committed to enter the dauer stage rather than the L3 stage [17]. At all other stages in the *daf-7* mutant, including in the dauer stage, GFP::DAF-16 was largely excluded from the nucleus Figure 3d. This observation suggests that nuclear localization of DAF-16 is an important effector in the DAF-7 TGF-β signaling pathway as well. Indeed, although *daf-16*; *daf-7* double mutants are dauer arrest constitutive, the dauers that result are nevertheless incomplete. They lack pharyngeal constriction and form indistinct alae [8].

Our result indicates that the *daf-7* TGF-β signaling pathway crosstalks with the insulin pathway at a point upstream of DAF-16 nuclear localization. *daf-7* TGF-β signaling could act anywhere in the insulin signaling pathway, from the expression or secretion of any of the worm insulins to DAF-16 itself. For example, *daf-7* could cause a surge of *ins-1* expression in the L2d stage, which, as an antagonist, would shut down the *daf-2* pathway [18]. Alternatively, in cultured mammalian cells, TGF-β treatments lead to a rapid downregulation of PTEN transcript accumulation [19]. A similar mechanism could operate in the worm: loss of *daf-7* TGF-β signaling may increase *daf-18* PTEN expression at the L2d stage, causing a reduction in insulin signaling and AKT-(1,2) kinase activity, to affect DAF-16 nuclear accumulation. We stress that whatever the mechanism connecting *daf-7* to DAF-16 localization may be, it functions only at the L2d stage. This explains why mutations in the TGF-β pathway are synergistic with those in the insulin pathway only in the dauer formation, but not the longevity, phenotype.

Given the conservation that has been observed between worm and mammalian insulin signaling pathways, and given that mammalian GDF-8 (accession number NM010834) and GDF-11 (accession number AF028337) are candidate DAF-7 orthologs, it is possible that these TGF-β signals may also interact with insulin signaling in mammals.

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